

## THE COLORIMETRIC DETERMINATION OF THE SERUM PROTEINS.

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In connection with the study of the state of the calcium of the blood, we desired a rapid method for determining serum proteins. Wu (1) and Wu and Ling (2) have published a method for the determination of plasma proteins based on the color developed with Folin's phenol reagent. The method as described by these authors is not very satisfactory due to the formation of turbid precipitates and the slow development of the color. Very recently Folin and Ciocalteau (3) have improved the phenol reagent by the addition of lithium sulfate. This permits the use of greater amounts of phenol reagent and sodium hydroxide without any resulting turbidity. By using more of the reagent the color development takes place far more rapidly and the amount of color is markedly increased. In connection with this improvement in the phenol reagent it seemed to us that a simplification could be obtained in the determination of serum proteins by adopting essentially Howe's method (4) of salting out the globulin with sodium sulfate solution. The sodium sulfate was found to have almost no effect on the color developed so the albumin can be determined directly on an aliquot of the filtrate. We were able in this way to work out a satisfactory method for the analysis of the albumin and globulin, making use of 0.5 cc. of serum.

*Reagents.*—The reagents required are 22.5 per cent sodium sulfate solution prepared from the anhydrous salt, 5 N sodium hydroxide, a standard tyrosine solution containing 200 mg. of pure dry tyrosine in 1 liter of approximately 0.1 N hydrochloric acid, and Folin's phenol reagent. To keep all the sodium sulfate in solution, it is necessary to keep this reagent in an incubator.

*Analytical Procedure.*—Pipette 0.5 cc. of serum from a calibrated pipette into a 15 or 20 cc. test-tube. Add exactly 9.5 cc. of 22.5 per cent sodium sulfate solution with a pipette of that volume or from a burette. Agitate thoroughly and set aside for about 2 hours in an incubator at 37° to allow coagulation of the globulin. At the end of this period, filter into another test-tube, using a fairly retentive filter paper (Whatman No. 42 is satisfactory). Examine to see that the filtrate is clear, if not, pour back on the filter paper. After the filtering is nearly complete, remove the tube containing the filtrate to be used for albumin analysis. The residue of globulin in the test-tube in which the precipitation was carried out is now washed onto the filter paper by two washings with 3 cc. each of sodium sulfate solution.

The globulin precipitate is then washed twice more with 3 cc. portions of sodium sulfate solution. The two protein fractions are now estimated according to the following procedure.

*Globulin.*—The funnel and filter paper containing the globulin precipitate are transferred to a 50 cc. volumetric flask, a small hole is punched in the bottom of the filter paper with a wire or drawn out glass rod, and the globulin dissolved and washed into the volumetric flask by a stream of approximately 0.01 N sodium hydroxide from a wash bottle. The washing is completed with distilled water until the flask is about half full. The filter paper is then unfolded and examined to see that all the globulin is dissolved. To the flask there are now added 2 cc. of 5 N NaOH and 3 cc. of the phenol reagent. The flask must be agitated while the phenol reagent is being added to prevent a large local excess which may result in a turbid precipitate. The flask is now filled to the mark with distilled water and the contents thoroughly mixed. A standard is prepared at the same time by pipetting 4 cc. of the standard tyrosine solution into another 50 cc. volumetric flask, adding about 25 cc. of water, then 2 cc. of sodium hydroxide, and 3 cc. of phenol reagent, agitating the contents of the flask during the course of adding the reagents, and finally filling with water to the graduation mark. After this has stood for from 5 to 10 minutes to insure full color development, the standard is set at the 20 cc. mark on the colorimeter and the unknown compared against it.

*Albumin.*—For the determination of the albumin, a 5 cc. aliquot

of the filtrate is pipetted into a 50 cc. volumetric flask, about 25 cc. of water are added, then 2 cc. of 5 N sodium hydroxide and 3 cc. of the phenol reagent. The flask is then filled with distilled water to volume and the contents thoroughly mixed. A standard is prepared at the same time with 4 cc. of standard tyrosine solution. After 5 to 10 minutes the colors are read with the standard set at the 20 mm. mark on the colorimeter.

*Total Serum Protein.*—If no upset of the normal albumin to globulin ratio is suspected, determination of total serum protein, may in many cases, be sufficient. This can be carried out according to the procedure given above, with either 0.2 cc. of whole serum or perhaps more accurately a 2 cc. aliquot of 1 cc. of serum that has been diluted in a 10 cc. volumetric flask. The comparison is carried out in the usual manner against either 4 or 5 cc. of standard tyrosine solution. This determination of total protein gives the possibility of determining the globulin, not directly, but by subtracting the value of the albumin fraction from the total protein. It is more accurate however to determine both albumin and globulin separately as described above.

#### DISCUSSION.

Some precautions need to be observed to obtain correct analytical results. The color obtained with the phenol reagent is dependent on the age of the serum sample. The amount of color obtained decreases with time as the serum stands. On this account freshly obtained sera that have stood no longer than overnight should be used for the analysis. The alkali and phenol reagent are to be in the proportions recommended. These amounts were found to give optimum color development under the conditions of the analytical method. In tests, made on the standard tyrosine solution, with the above amounts of alkali and phenol reagent, there was obtained a proportionality of color to within 1 per cent, when a solution containing 4 cc. of tyrosine standard was compared against solutions containing 3 and 5 cc. of tyrosine standard. When a similar test, with 2 cc. of alkali and 2 cc. of phenol reagent, was carried out, the solution with 3 cc. of tyrosine standard gave 5 per cent more than proportional color while the 5 cc. tyrosine sample gave 6 per cent too low a color.

Howe (4) found that the salting out of the globulin was constant

when the sodium sulfate concentration was kept between 21 and 22 per cent and the dilution between 10 and 30 volumes. In his micro method (5) 0.5 cc. of serum is diluted to 15 cc. with salt solution. In our procedure it is more convenient to keep the volume down to 10 cc. That there is no objection to changing from the dilution used by Howe to that employed by us is shown by Howe's findings and also by some tests carried out by us. In agreement with Howe, it was found that the same amount of globulin, as shown by the color obtained, was salted out on a 10-, 20-, or 30-fold dilution of the serum.

The effect of sodium sulfate on the color was tested by carrying out parallel tests on diluted serum, with and without sodium sulfate in amounts equivalent to that present in the albumin analysis. The salt was found to give a decrease in color of about 1 per cent. Similarly, since the albumin filtrate contains some of the original liquid of the serum, tests were carried out to find the amount of color due to the protein-free serum filtrate. Serum samples of 2 cc. were precipitated with trichloroacetic acid. The acid of the filtrate was then neutralized and the color determined by adding alkali and phenol reagent and comparing against a standard tyrosine solution. From the color obtained it was calculated that the color due to the protein-free filtrate in the albumin aliquot amounts to about 2 per cent of the total color. It is to be seen that the diminution in color because of the sodium sulfate is about compensated by the color due to the protein-free filtrate.

*Determination of Tyrosine Equivalents of Serum Proteins.*—The tyrosine equivalents of the serum proteins were determined for human blood, with pooled samples of serum, by making parallel determinations by the colorimetric method and by Kjeldahl analysis. Total protein of the serum was determined on 2 cc. samples by precipitating with 5 per cent trichloroacetic acid solution, washing, and determining the nitrogen. Globulin was determined on 5 cc. serum samples by salting out with sodium sulfate and determining the nitrogen by the Kjeldahl method. The analyses were carried out in duplicate. The albumin nitrogen was obtained by subtracting the globulin from the total protein. The protein fractions were then calculated by multiplying the nitrogen by the factor 6.25 as adopted by Wu. The average value

of the factors obtained, in terms of the mg. of protein that give a color equivalent to that given by 1 mg. of tyrosine are: total protein, 16.0; albumin, 16.6; globulin, 14.4.

*Calculations.*—The general formula for calculating the percentage of protein from the colorimetric reading is:

$$\frac{R}{X} \times T \times \frac{100}{S} \times \frac{f}{1000} = \text{protein (in per cent)}$$

TABLE I.  
*Colorimetric Analysis of Serum Proteins.\**

Subject.	Diagnosis.	Albumin.	Globulin.	Ratio, albumin globulin
		<i>per cent</i>	<i>per cent</i>	
Ne	Normal.	4.5	2.2	2.05
Co	"	5.0	2.2	2.27
Se	"	5.3	2.4	2.20
Mrs. Ga	" pregnancy.	3.8	1.9	2.00
" La	" "	4.0	2.1	1.90
Sw	Jaundice.	4.1	2.4	1.70
Fra	"	3.6	2.6	1.38
Cant	" (terminal stage).	3.4	2.9	1.17
C	" and gaseous infection.	3.8	1.7	2.20
Ho	Hyperthyroidism.	4.6	2.0	2.30
Ms	Hyperplasia of thyroid.	4.4	2.7	1.63
Sa	Carcinoma of stomach.	4.2	2.3	1.82
Du	" " tongue.	5.1	2.3	2.22
Mrs. Swa	Sarcoma.	4.0	1.6	2.50
Me	Infectious arthritis.	5.3	2.3	2.30
Mrs. An	Arthritis.	5.3	2.5	2.12

\* These results have been obtained in cooperation with the Department of Medicine of the University of California Medical School.

In the formula  $R$  is the point at which the standard is set,  $X$  the reading of the unknown,  $T$  is the mg. of tyrosine in the standard solution,  $S$  is the aliquot of serum used, and  $f$  the factor for the particular protein fraction being analyzed. For the albumin and globulin determinations, the colorimetric standard is set at 20 mm. and the amount of standard tyrosine solution contains 0.8 mg. The globulin represents a 0.5 cc. aliquot of serum and the albumin half of this value.

In our hands the method gave results accurate to about 5 per cent. For clinical use, it offers a rapid and relatively simple means of determining the serum proteins. A few typical values from our analytical results are given in Table I.

#### SUMMARY.

A colorimetric method is described for determining serum proteins based on the color developed with Folin's phenol reagent and by Howe's method of separating the proteins by sodium sulfate.

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